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14. ABSTRACT

Inflammatory bowel disease is associated with the relative increase in the population of *Proteobacteria* compared to other bacterial phyla, which in turn might be linked to cancer predisposition. Using a *Drosophila melanogaster* model of intestinal infection we have investigated various human intestinal bacteria, including *Proteobacteria* and screened for the inflammatory responses they elicit individually or in binary combinations. We find that some highly inflammatory bacterial strains loose their inflammatory effect when in combination with other highly inflammatory strains. Thus specific bacterial compositions rather than mere presence or absence of bacterial species dictates the degree of bacterially-driven intestinal inflammation.

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Inflammation, intestinal bacteria, infection, cancer

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INTRODUCTION

Until now it remains unknown if a relative increase in the population of *Proteobacteria* in comparison with other abundant bacterial phyla is a cause or a result of inflammatory bowel disease (IBD). Our study aimed to assess systematically for the first time the differences in inflammatory potential of a broad array of human intestinal bacteria in order to identify specific bacterial species and combinations thereof for their ability to promote intestinal inflammation. Using a *Drosophila melanogaster* model of intestinal infection we have investigated various human intestinal bacteria, predominantly *Proteobacteria* which can easily colonize the *Drosophila* intestine. We assessed the inflammatory responses elicited by individual bacterial strains. To challenge the assumption that highly inflammatory species will also be virulent in the presence of other inflammatory bacteria we prioritized assessments of combinations of highly inflammatory stains in an attempt to identify competitive bacterial interactions. We find bacterial species that while high inflammatory when mono-associated with the host, they loose their damaging ability when in combination with other damaging strains. Thus specific bacterial combinations rather than the presence of particular species *per se* result in a low intestinal inflammation status in our experimental model.

BODY

Intestinal microbiota composition is critical in intestinal inflammation and their perturbations are linked to Inflammatory Bowel Disease (IBD). Other than *Helicobacter pylori* in gastric ulcer and cancer, no other bacterial species has been accepted as a causative agent of chronic gastrointestinal inflammation and cancer (Zabaleta, 2012). IBD is a chronic disease characterized by inflammation of the gastrointestinal tract and colon with an increased risk for colon cancer (Tanaka, 2012). Evidence suggests that intestinal microbiota is a very important factor in IBD (Kaser et al., 2009). Earlier work from us and other labs has addressed the inflammatory role of bacteria, including human intestinal bacteria, in the intestinal epithelium of *Drosophila melanogaster* (Apidianakis et al., 2011). *Drosophila* intestinal pathology of the midgut provides an ideal environment for inflammation research as the intestinal stem cell mitosis is activated following induction of cytokines and growth factors to sustain cellular homeostasis and can be easily quantified through mitotic staining of the gut. For example, we have previously found increased intestinal mitosis upon infection with *Pseudomonas aeruginosa*, a human opportunistic pathogen in *Drosophila* (Apidianakis et al., 2009).
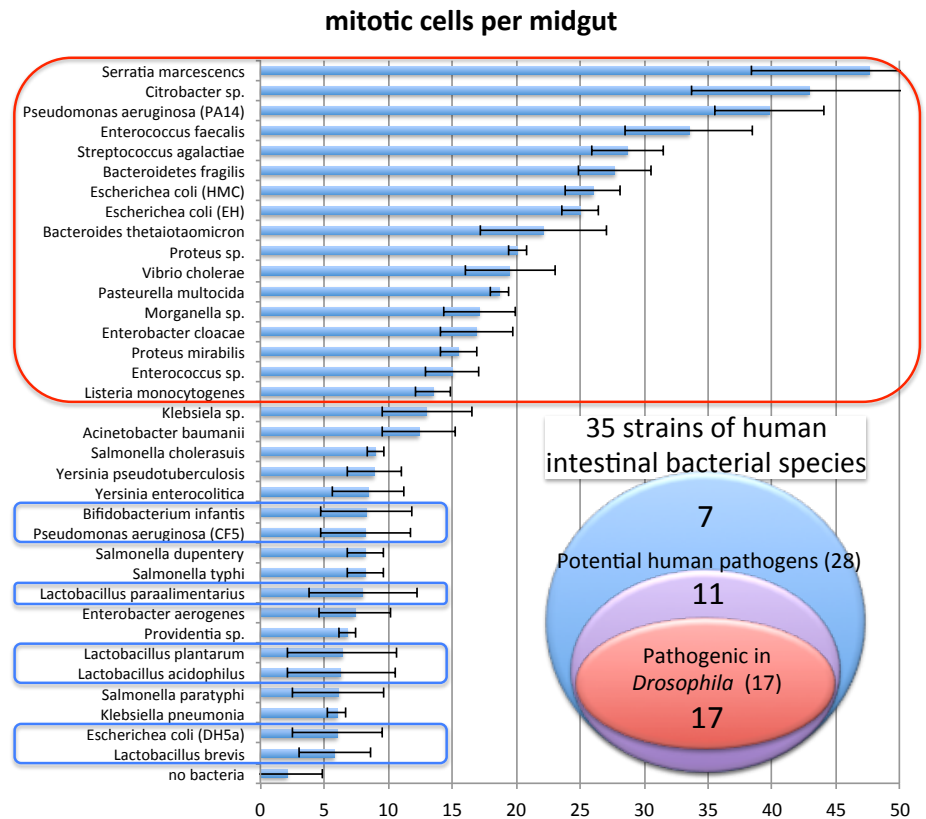

Figure depicts 17 human intestinal bacteria (red frame) found to be highly inflammatory and 7 low inflammatory strains (blue frame) in our model of intestinal inflammation in *Drosophila*. Mitotic index in the *Drosophila* midgut is indicative of the potential of these microbes to induce intestinal inflammation.

According to our **Statement of Work (SOW)** (please refer to the end of this section for the pertinent Task 1 and Task 2, as they were originally proposed) we aimed to assess the effect of human intestinal *Proteobacteria* and other intestinal bacterial species on epithelial inflammation. The human intestine contains mainly anaerobic bacterial species belonging to the phyla *Firmicures* and *Bacteroidetes* and to lesser extent aerobic *Proteobacteria* species. Nevertheless, the latter are more likely to survive in the aerobic environment of the *Drosophila* intestine. In addition, *Proteobacteria* are hypothesized to be instigators in IBD. Thus instead of selecting an equal number of 32 *Proteobacteria* vs 32 *Firmicutes* and *Bacteroides* bacterial strains, as we had proposed in **Task 1**, we assessed 29 of the former and 6 of the latter, all of which are able to colonize the *Drosophila* intestine. The most important part of Task 1 was to assess all selected human strains for their inflammatory potential. We measured the average and standard deviation of mitgut mitosis from ~20 midguts per bacterial strain (**Figure 1**, unpublished). While intestinal bacteria that cause disease in humans can occasionally cause disease in other organisms, the extent to which a *Drosophila* host can predict the pathogenic potential of human intestinal bacteria is not known. Accordingly, we found a good correlation between human intestinal bacteria that induce significant regeneration, i.e. mitosis, in the *Drosophila* intestine and their known potential to be pathogenic in humans. Out of our diverse panel of 35 culturable human intestinal bacterial strains, 17 can significantly increase intestinal cell mitosis, a quantitative marker of intestinal inflammation (**Figure**

1). Strikingly, all 17 strains have been previously associated with opportunistic and other infections in humans, yet none of the 7 strains considered non-pathogenic in humans induced significant intestinal mitosis in flies (**Figure 1**). Thus there is a high correlation between potential pathogenicity in humans and enterocyte damage and regeneration/mitosis in the *Drosophila* intestine.

With the 35 strains in hand we worked on the most laborious and time consuming part of SOW, the assessment of the inflammatory potential of the strains introduced in binary combinations in the *Drosophila* intestine. In our original **Task 2** we proposed to assess the selected *Proteobacteria* in 1:1 combinations with the selected Firmicutes/Bacteroidetes species. Nevertheless, our strain selection finally contained only a few *Firmicutes/Bacteroidetes* species, such as *Lactobacillus* and *Bifidobacteria* species (**Figure 1**), most of which are considered to be beneficial to humans. In addition, we had to amend our protocol so as to increase the number of midguts we assess from 5 to ~12 per condition, a necessary change to make our results more reliable. Finally, we found it necessary to reproduce our findings in order to validate our results within the time frame of this award. Thus, we prioritized the combinatorial assessments proposed in Task 2, so as to combine 7 strains exhibiting the highest inflammation in mono-associated *Drosophila* with 20 strains exhibiting extreme inflammation (10 highest plus 10 lowest, according to the ranking shown in **Figure 1**). We found that 48 strain combinations reduce the inflammatory potential of the top 7 inflammatory strains (**Table 1**, unpublished). Of those we verified 13 combinations i.e we observed qualitatively similar results upon repetition of the experiments (**Table 1**). For example, the highly inflammatory species *Pseudomonas aeruginosa* loose its inflammatory potential when in combination with the equally inflammatory species *Escherichia coli, Vibrio colerae* and *Bacteroides fragilis*. Similarly, the highly inflammatory species *Enterococcus faecalis* loose its inflammatory potential when in combination with the equally inflammatory species *Vibrio colerae* and *Bacteroides fragilis*. **These findings are of great importance because they suggest that bacterial combinations can exert mutual antagonistic effects on intestinal inflammation.** The information that even inflammatory bacterial strains may reduce the inflammatory potential of other bacteria could be exploited by clinicians in the prediction or treatment of intestinal inflammation. Pertinent adjustments in the microbiota of patients with intestinal inflammation may help to improve treatments against Inflammatory Bowel Disease (IBD), but also to reduce the risk of inflammation-driven tumorigenesis (Tanaka, 2012).

Table 1. Combinations of highly inflammatory species with either high (black) or low (green) inflammatory ones resulting in low inflammation

Citrobacter
- E.coli
- *Proteus*
- E. aerogenes
- Salmonella paratyphi
- Providencia
- L. plantarum*
- L. brevis*

B. fragilis
- Serratia
- *P. aeruginosa*
- E. faecalis
- *S. agalacteae*
- E. coli
- *Proteus*
- V. colerae
- Y. pseudotuberculosis
- B. infantis*
- L. plantarum*
- L. acidophilus*

P. aeruginosa
- *E.coli*
- *V. colerae*
- *B. fragilis*
- B. thetaiotaomicron
- E. aerogenes
- K. pneumoniae
- Y. pseudotuberculosis
- B. infantis*

E.coli
- V. colerae
- P. maltocida
- ??

E. faecalis
- *V. colerae*
- *B. fragilis*
- B. thetaiotaomicron
- K. pneumoniae
- Providencia
- B. infantis*
- L. plantarum*
- L. paralimentarium*

Serratia
- *E. coli*
- Citrobacter
- *E. faecalis*
- V. colerae
- ??

S. agalacteae
- *P. aeruginosa*
- Proteus
- V. colerae
- B. fragilis
- *B. thetaiotaomicron*
- Y. pseudotuberculosis
- Providencia
- L. acidofilus*

BLACK=inflammatory in flies (underlined=verified)
GREEN=non-inflammatory in flies
*known as beneficial in humans

Originally proposed SOW Tasks:

Task 1. Select 32 Proteobacteria and 32 Firmicutes/Bacteroidetes species that are can be transmitted to the *Drosophila* intestine upon feeding and assess if they induce intestinal inflammation (*months 0-2*):
1a. Collect 40 Proteobactia and 60 Firmicutes/Bacteroidetes species that are published to be common in the human intestine and have been cultured before (*months 0-1*).
1b. Out of these 100 species we will select 32 Proteobacteria and 32 Firmicutes/Bacteroidetes species that are can be transmitted to the *Drosophila* intestine upon feeding (*months 1-2*).

1c. Assess if the selected 64 species can induce intestinal inflammation in a *Drosophila* model of intestinal inflammation (*months 1-2*).

Task 2. Assess the selected Proteobacteria in 1:1 combinations with the selected Firmicutes/Bacteroidetes species (32x32=1024 combinations) for their ability to induce intestinal inflammation in *Drosophila* (*months 3-12*):
2a. Feed *Drosophila* with 1024 combinations of bacteria for 5 days, dissect out, fix and mount in a microscope slide 5 of their intestines (*months 3-12*).
2b. Measure the average number of progenitor cells per bacterial stain combination and categorize accordingly (*months 3-12*).

KEY RESEARCH ACCOMPLISHMENTS

• We selected a diverse panel of 35 culturable strains of human intestinal bacterial species, able to colonize the *Drosophila* intestine and assessed their inflammatory potential (**Figure 1**).

• Figure 1 data analysis shows that there is a high correlation between intestinal mitosis in the *Drosophila* midgut and the know pathogenicity potential in humans of the intestinal bacterial strains assessed.

• We assessed binary combinations of highly inflammatory strains and other strains (as shown in **Table 1**) to find that highly inflammatory species, such as *Pseudomonas aeruginosa* and *Enterococcus faecalis* loose their inflammatory potential when in combination with the equally inflammatory species *Vibrio colerae* and *Bacteroides fragilis*.

• Table 1 data analysis shows that bacterial combinations can exert mutual antagonistic effects on each other to diminish their impact on intestinal inflammation.

REPORTABLE OUTCOMES

Manuscripts stemming from this work will be accomplished in the future due to the short duration (1 year) and nature (screening) of this study. Nevertheless, below we list all the significant outcomes of our work:

1. Employment of Georgios Apidianakis (PI) for 1 year
2. Employment of Jianxin He (postdoctoral researcher) for 1 year
3. Data presentation (poster) by Stavria Panayidou (PhD student in Apidianakis lab) in InflaCancer meeting in Greece, September 2012
4. PhD thesis project and mobility grant ERASMUS to Theodoulakis Christofi (PhD student in Apidianakis lab)
5. Career Integration Grant (Marie Curie) award to Georgios Apidianakis based on preliminary results stemming from this work.

CONCLUSSIONS
For an extensive discussion of conclusions please read the 2nd half of page 6.

"So what section": With our research we shift the focus from potential individual bacterial pathogens to combinations of potential bacterial pathogens as a criterion for intestinal inflammation. In this regard, we pave the way for further studies of intestinal inflammation and cancer to focus on specific bacterial interactions in order to assess combinations of intestinal bacteria rather than mere presence or absence of potentially harmful bacteria in the human intestine.

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